INTERNATIONAL APPLICATION PUBLISHED
IN ACCORDANCE WITH THE PCT

(19) **World Organization of Intellectual Property**, International Office

(43) **International Publication Date 19 April 2001**

(10) **International Publication No.: WO 01/27295 A1**

---

(51) **International Patent Classification[7]: C12N 15/75**, A61K 39/02, C12N 1/21

(21) **International File No.:** PCT/DE00/03629

(22) **International Application Date:** 13 October 2000

(25) **Language of Submittal:** German

(26) **Language of Publication:** German

(30) **Information on Priority:**
199 49 594.7 14 October 1999 DE

(71) **Applicant** *(for all designated countries except US)*: **DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES OFFENTLICHEN RECHTS** [DE/DE]; Im neuenheimer Feld 280, 69120 Heidelberg (DE).

(72) **Inventor; and**
(75) **Inventor/Application** *(only for US)*: **SCHADENDORF, Dirk** [DE/DE]; Weberstrasse 3, 68165 Mannheim (DE). **PASCHEN, Annette** [DE/DE]; Scheffelstrasse 90, 68259 Mannheim (DE). **CHAKRABORTY, Trinad** [SG/DE]; Seltersweg 85, 35390 Giessen (DE). **DOMANN, Eugen** [DE/DE]; Dreispitz 23, 35444 Biebertal (DE).

(74) **Attorney: SCHÜSSLER, Andrea**; Huber & Schüssler, Truderinger Strasse 246, 81822 Munich (DE)

(81) **Designated Countries** *(national)*: AU, CA JP, NZ, US.

(84) **Designated Countries** *(regional)*: European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
- *With international search report.*
- *Before deadline for changes to the claims; publication will be repeated if changes are made.*

For an explanation of the two-letter codes and the other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" at the start of each regular issue of the PCT Gazette.

---

(54) **Title:** RECOMBINANT ATTENUATED LISTERIAS FOR IMMUNOTHERAPY

(57) **Abstract:** [in English]

Recombinant Attenuated Listerias for Immunotherapy

The present invention concerns Listeria expression vectors, which permit the expression of the human tumor-associated antigens tyrosinase, Trp-1, MelanA/MART-1 and Trp-2, as well as attenuated Listeria bacteria containing these expression vectors, preferably bacteria of the strain Listeria monocytogenes. These bacteria can be used for prophylactic, adjuvant or therapeutic immunotherapy, for example, in treatment of malignant melanoma.

At present, tumor therapy is still primarily based on the three pillars: surgery, chemotherapy, adjuvant chemo and radiation therapy. However, these therapies have the following serious drawbacks: (a) they are of little effect in the metastasizing stage of the disease or as preventive therapy after removal of the primary tumor, i.e., a cure is no longer possible in the metastasizing stage, (b) they are very inadequate in regard to the clinical response, the length of the relapse-free interval, the overall survival time, and the quality of life of the patient, and (c) they have a number of sometimes severe side effects, for example, a considerable damaging of normal tissue. Furthermore, there have been no preventive opportunities thus far, such as might involve a "protective inoculation." Yet this would be very desirable in regard to malignant melanoma.

Thus, the technical problem essentially underlying the invention is to furnish means of tumor therapy, especially for treatment of malignant melanoma, that do not have the above described drawbacks of the present therapy methods, in particular, such as enable a preventive application, are effective as adjuvant after removal of a primary tumor, or are of therapeutic benefit in the stage of distant metastasis.

The solution of this technical problem was accomplished by furnishing the embodiments characterized in the patent claims.

The Listeria expression vectors and the recombinant attenuated Listeria bacteria of the invention are effective constructions for gene therapy, which can be used for preventive or as part of an adjuvant or therapeutic tumor fighting, especially for malignant melanoma. With a preferably oral immunization, a tumor-specific immune response can be created, i.e., a targeted fighting of the tumor cells can be achieved through the body's own cellular immune system. Furthermore, this treatment if necessary can be combined with treatment methods such as chemotherapy or radiation therapy, but preferably it replaces the latter forms of therapy.

The present invention is based on the finding that an immunization, preferably oral immunization, making use of the invented recombinant attenuated Listerias as a synthesis and transport vehicle for tumor-associated antigens makes possible a preventive or therapeutic treatment of tumors. The expression of the individual tumor-associated antigens preferably occurs as fusion proteins in which, for example, a Listeria-specific signal sequence is fused to the N-termination of the antigen. After being expressed, these fusion proteins are exported from the bacterial cells into the surroundings. After oral application, the bacteria pass through the mucosal epithelium of the intestinal tract in the region of Peyer's patches and are taken up by the antigen-presenting cells (APCs) of the immune system located there by phagocytosis. Thus, the Listeria at first are present in the phagosome of the infected cells, into which they secrete the fusion proteins, which are thus available to a processing in order to generate HLA Class I and HLA Class II peptides. Through the

natural infection cycle, both the wild-type Listeria bacterium and certain attenuated mutants (insofar as they have no deletion of the hly gene) can cross into the cytosol of the cell. The fusion proteins secreted into the cytosol, in turn, are available to a processing to generate HLA Class I peptides. Thus, the peptides generated in both compartments of the cell (phagosome and cytosol) are available for loading onto HLA I or HLA Class II molecules. The peptide-HLA complex is presented on the cell surface of the APCs and a specific T-cell response (CD4+ and CD8+ T-cells) is induced against the expressed tumor-associated antigens, i.e., a cellular cytotoxic immune response of the body's own immune system to a tumor. Hence, the tumor cells are specifically recognized as abnormal and are killed. The benefits of this procedure include the fact that the body's own immune system is specifically mobilized to destroy the tumor. It constitutes a simple immunization technique, since the APCs contain the tumor-associated antigens by means of a bacterial infection, i.e., the invented technique requires no labor-intensive ex vivo modification of autologous APCs, since a naturally occurring infection process is utilized to modify the cells of the immune system.

Thus, the present invention concerns a Listeria expression vector for immunotherapy, wherein the Listeria expression vector comprises the following functionally related DNA sequences: (a) a promotor active in Listeria; and (b) a DNA sequence coding for human tyrosinase, Trp-1, MelanA/MART-1 or Trp-2 or an antigen epitope derived from it.

The term used here "promotor active in Listeria" refers to all promotors which allow for expression of the tumor-associated antigens in Listeria. Preferably, it involves promotors of Listeria monocytogenes genes, for example, constitutive promotors or those which are activated under the conditions of the infection. Especially preferred are promotors which lead to a strong expression of the desired antigen. The term also refers to promotor fragments or promotors with modified sequences that are still biologically active. In one preferred embodiment, the promotor for the Listeria expression vectors is a promotor of the hly, actA, plcA, plcB or mpl gene, each of which are active under the conditions of the infection and code for the Listeria proteins hemolysin, ActA, phosphotidylinositol-specific phospholipase C, phosphatidylcholine-specific phospholipase C or metalloprotease. These promotors have already been described at length in the literature:

- actA/plcB promotor: Domann, et al., (1992), EMBO J. 11: 1981-1990 (The transcription of the actA and the plcB gene is controlled by a common promotor)

- hly promotor: Domann, et al., (1989), Nucleic Acids Res. 17: 6406

- plcA promotor: Domann, et al., (1991), Mol. Microbiol. 5: 361-366

- mpl promotor: Domann, et al., (1991), Infect. Immun. 59: 65-72

The term used here "DNA sequence coding for human tyrosinase, Trp-1, MelanA/MART-1 or Trp-2" concerns every DNA sequence that codes the native protein in full or partially. These DNA sequences and the derived amino acid sequences are described, for example, in:
human tyrosinase gene: Genbank Accession No.: M37160
human trp-1 gene: Genbank Accession No. AF001295
human trp-2 gene: Genbank Accession No. D17547

human MelanA/MART-1 gene: Genbank Accession No. U06452
Moreover, refer to Figures 1-4 for this.

The antigens tyrosinase, Trp-1, Trp-2 and MelanA/MART-1 are differentiation antigens of melanocyte origin. Since these antigens are expressed exclusively in melanocyte cells (melanocytes) as part of the melanogenesis process, they are exceptionally well suited to generate a specific immune response to melanoma cells. These differentiation antigens, furthermore, have the advantage that they are expressed by up to 100% of the cells of a pigmented tumor (melanoma), whereas only around 50% of the melanomas express cancer-testis antigens, such as MAGE-1. The enzymes tyrosinase, Trp-1, Trp-2 catalyze the process of pigment formation (malanin [sic?] synthesis). The biosynthesis takes place in the specific organelles, the melanosomes, in whose matrix the MelanA/MART-1 protein is also localized.

The term "DNA sequence coding for human...," moreover, also concerns DNA sequences which code for forms of human tyrosinase, Trp-1, MelanA/MART-1 and Trp-2 which have changes compared to the native form, i.e., such as deletions, additions or exchanges of one or more amino acids and/or modified amino acid(s) or the attachment of a ubiquitin residue or altered oligosaccharide side chains, while their antigen properties remain fully or partly or in the desired manner, i.e., they may have the properties described in the following examples with respect to the treatment of a tumor. The exchanges include, preferably, "conservative" exchanges of amino acid residues, i.e., exchanges for biologically similar residues, e.g., the substitution of a hydrophobic residue (e.g., isoleucine, valine, leucine, methionine) for another hydrophobic residue, or the substitution of a polar residue for another polar residue (e.g., arginine for lysine, glutaminic acid for asparaginic acid, etc.). The exchanges also include "nonconservative" exchanges, which can preserve or even strengthen the antigen properties of the proteins or individual derived protein fragments (peptides). This can completely alter the biological or enzymatic activity of the native protein. Deletions can result in creation of molecules having a distinctly smaller size, i.e., lacking amino acids at the N or C-termination. The aforesaid variants also involve variants which have a better activity at tumor fighting than the original form. Techniques for creating the aforesaid changes in the amino acid sequence or corresponding nucleic acid sequence are familiar to the practitioner and are described in standard molecular biology texts, such as Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). The practitioner is also able to determine whether a protein or peptide encoded by such an altered nucleic acid sequence still has the desired antigen properties of tyrosinase, Trp-1, MelanA/MART-1 or Trp-2 (in whole or in part). These antigen properties can be ascertained for the proteins/peptides by stimulating antigen-specific cytotoxic T-cell lines. In the case of peptides, one can also verify their HLA-binding properties in the context of FACS analysis. Preferably, the DNA sequences encoding the tyrosinase, Trp-1, MelanA/MART-1, Trp-2 or the above variants should have a transcription termination sequence and a translation termination sequence to ensure a stable and correct transcription and translation.

General methods known in the profession can be used to construct the Listeria expression vectors of the invention, including those for ligation of the fragments for the promotor and the tumor-associated antigens and insertion into the vector. These methods include, for example, in vitro recombination techniques, synthetic methods, as well as in vivo recombination methods, like those described in Ausubel and Frederick (1991), Current Protocols in Molecular Biology (J. Wiley &

Sons, New York).

The most preferable are Listeria expression vectors in which the DNA sequence coding for human tyrosinase, Trp-1, MelanA/MART-1 or Trp-2 is linked to a DNA sequence encoding a Listeria protein (fragment) so that a fusion protein is encoded. Preferably, the portion coming from the Listeria protein represents the N-terminal portion of the fusion protein. Various Listeria proteins or fragments thereof are suitable for construction of this fusion protein, for example, the genes already disclosed with regard to the Listeria promotors. Even more preferred are Listeria expression vectors in which the Listeria protein component of the fusion protein comes from a protein involved in the lysis of the host vacuoles or movements of the bacteria in the host cell, preferably Listeriolysin O (lysis), ActA (intracellular movement) or PI-PLC (lysis). The advantage of using Listeriolysin O, a Listeria-phospholipase or the ActA protein for construction of fusion proteins is that these proteins are secreted. In event of an infection, these fusion proteins therefore preferentially reach the phagolysosome or the cytosol of the infected cell, i.e., the cell compartment where the generation of HLA Class I and HLA Class II presented peptides occurs. The fusion proteins can preferably have the following appearance:

a) only the secretory signal sequence of a Listeria-specific protein is fused to the antigen
b) the secretory signal sequence including a segment of the adjoining native protein sequence is fused to the antigen,
c) a short fragment of the antigen is incorporated into the sequence of the mentioned Listeria protein while maintaining the reading frame.

Moreover, the present invention concerns Listeria expression vectors in which the Listeria protein component of the fusion protein comprises a signal sequence of a secreted Listeria protein. Preferably, the signal sequence comes from Listeria-hemolysin, a Listeria phospholipase or the ActA protein.

The starting vector for the construction of the Listeria expression vector of the invention is any vector which leads to the expression of the desired antigens in Listeria. It can be an autosomal vector or one that is stably inserted into the Listeria genome. Preferably, the starting vector is a "shuttle" vector, which can be replicated in a further host, such as E. coli. Such vectors are, for example, pKSV7 (Frankel, et al., 1995, J. Immunol. 155: 4775-4782), pCGU34 (Paglia, et al., 1997, Eur. J. Immunol. 27: 1570-1575), pAUL-A (Niebuhr, et al., 1997, EMBO J. 16: 5444-5445) and pLIGA160.

The present invention also concerns recombinant, attenuated Listeria bacteria containing the Listeria expression vectors of the invention (integrated autosomally or stably into the genome, for example, through homologous recombination), preferably Listeria monocytogenes or Listeria innocua, the latter being especially suitable to strengthening an immune response. The choice of suitable Listeria can be made by the practitioner using conventional criteria for the use of bacteria in vaccination, i.e., the Listeria used for purposes of the invention should have immunogenicity, yet be sufficiently attenuated to permit a safe use in humans. For this, it is necessary for the mutant phenotype of the Listerias to be absolutely stable, which is usually only possible by the creation of chromosomal deletions. Among the examples of attenuated mutants are those which are deficient in respect of propagation from one cell to another, actA-negative mutants which are deficient in

respect of intracellular growth, hly2-(Listerioloysin)-negative mutants, and mutants which are deficient at least in respect of a phospholipase gene (Guzman, et al., Infect. Immun. 63 (1995), 3665-3673). Examples of suitable Listeria strains include the Δmp12-mutants (Paglia, et al., Eur. J. Immunol. 27: 1570-1575). Suitable attenuated Listeria strains are also described in the international patent application PCT/EP98/08096. Methods for transformation of the aforesaid Listerias with the Listeria expression vectors of the invention, such as electroporation, for phenotypic selection of transformants, and for expression of the DNA sequences encoding the tumor-associated antigens (possibly as fusion proteins) making use of the above described Listeria expression vectors are familiar to the profession.

Finally, the present invention also concerns a pharmaceutical (inoculant) containing the recombinant, attenuated Listeria bacteria of the invention and its use for immunotherapy. This immunotherapy is suitable for treatment of pigmented types of tumors, preferably for treatment of malignant melanoma or malignant schwannoma (a subgroup of the neuroblastomas). These pharmaceuticals possibly also contain a pharmaceutically tolerable substrate. Suitable substrates and the formulation of such pharmaceuticals are familiar to the practitioner. Suitable substrates include, for example, phosphate-buffered salt solutions, water, emulsions, such as oil/water emulsions, wetting agents, sterile solutions, etc. The invented pharmaceutical can be dispensed orally, for example, in the form of an elixir, a capsule, or a suspension. The suitable dosage and the type of administration, preferably oral, intravenous, or intraperitoneal administration, will be determined by the treating physician and depend on various factors, such as the age, the gender, the weight of the patient, the stage and the severity of the tumor, the type of administration, etc. In any case, the administration must be done in an effective quantity, i.e., a quantity such that the tumor-associated antigen is expressed in a quantity which induces an immune response in the T-cells against the tumor-associated antigen, so that cells containing this antigen are destroyed. The pharmaceutical of the invention can be given either alone or in combination with other tumor therapies.

Expression vectors according to the invention have been deposited with the DSMZ (German Collection of Microorganisms and Cell Cultures), Mascheroder Weg 1b, Braunschweig, on 5 October 1999, in keeping with the provisions of the Budapest Treaty. The deposited specimens were assigned the following file numbers:
Escherichia coli XL2-Blue pLIGA-trp2 DSM 13072
Escherichia coli XL2-Blue pLIGA-tyro DSM 13073
Escherichia coli XL2-Blue pLIGA-trp1 DSM 13074

The figures further explain the invention.

Figure 1: Representation of the cDNA encoding region of human tyrosinase and the protein sequence derived from it

Figure 2: Representation of the cDNA encoding region of human Trp-1 and the protein sequence derived from it

Figure 3: Representation of the cDNA encoding region of human Trp-2 and the protein sequence derived from it

Figure 4: Representation of the cDNA encoding region of human MART-1/MelanA and the protein sequence derived from it

Figure 5: Analysis of the surface markers of infected dendritic cells. The expression of the surface markers of dendritic cells not infected with L. monocytogenes bacteria (thin lines) and that of infected dendritic cells (thick lines) was analyzed. Hatched gray histograms represent the controls.

The following examples illustrate the invention.

Example 1: Production of Two Different Listeria Expression Vectors Expressing Human Tyrosinase

At the 5' and 3' end of the cDNA (Gene Bank Access Number: M27160) encoding for human tumor-associated tyrosinase antigen, PCR (see Table 1) was used with the specific primers indicated in Table 1 and 2 (tyr-5/2-LIGA+tyr/3-LIGA; tyr/5-LIGA+tyr/3-LIGA) to introduce a NdeI and a SalI recognition sequence. The primer combination tyr-5/2-LIGA and tyr/3-LIGA (Table 1) was used to amplify the complete tyrosinase cDNA sequence. The primary combination tyr/5-LIGA and tyr/3-LIGA (Table 2) was used to amplify a 5'-deleted tyrosinase cDNA sequence. Both PCR amplificates were then treated as follows: Using the mentioned restriction intersections, the fragment was cloned into the vector pLIGA160 in frame downstream from the plasmid-encoded actA signal sequence. The expression of the resulting fusion gene is controlled by the plasmid-encoded actA-promotor (Domann, et al., 1992, EMBO J. 11: 1981-1990; Gene Bank Accession: X59723). The inserted DNA sequence and its transition points to the vector were sequenced as a check. The resulting expression vector is designated pLIGA-tyrof (coded total tyrosinase cDNA) or pLIGA-tyro (coded 5'-deleted tyrosinase cDNA). The latter was deposited with the DSMZ on October 5 as No. DSM 13073.

Example 2: Production of Two Different Listeria Expression Vectors Expressing the Human trp-1 Protein

At the 5' and 3' end of the cDNA (Gene Bank Access Number: AF001295) encoding for human tumor-associated Trp-1 antigen, PCR (see Table 1) was used with the specific primers indicated in Table 1 and 2 (trp1-5/2-LIGA+trp1/3-LIGA; trp1/5-LIGA+trp1/3-LIGA) to introduce a NdeI and a Bgl II recognition sequence. The primer combination trp1-5/2-LIGA and trp1/3-LIGA (Table 1) was used to amplify the complete trp-1 cDNA sequence. The primary combination trp1/5-LIGA and trp1/3-LIGA (Table 2) was used to amplify a 5'-deleted trp-1 cDNA sequence. Both PCR amplificates were then treated as follows: Using the mentioned restriction intersections, the fragment was cloned into the vector pLIGA160 in frame downstream from the plasmid-encoded actA signal sequence. The expression of the resulting fusion gene is controlled by the plasmid-encoded actA-promotor (Domann, et al., 1992, EMBO J. 11: 1981-1990; Gene Bank Accession: X59723). The inserted DNA sequence and its transition points to the vector were sequenced as a check. The resulting expression vector is designated pLIGA-trp1f (coded total trp-1 cDNA) or pLIGA-trp1 (coded 5'-deleted trp-1 cDNA). The latter was deposited with the DSMZ Braunschweig on 5 October 1999 as No. DSM 13074.

Example 3: Production of Two Different Listeria Expression Vectors Expressing the Human trp-2 Protein

At the 5' and 3' end of the cDNA (Gene Bank Access Number: D17547) encoding for human tumor-associated Trp-2 antigen, PCR (see Table 1) was used with the specific primers indicated in Table 1 and 2 (trp2-5/2-LIGA+trp2/3-LIGA; trp2/5-LIGA+trp2/3-LIGA) to introduce a NdeI and a Sal I recognition sequence. The primer combination trp2-5/2-LIGA and trp2/3-LIGA (Table 1) was used to amplify the complete trp-2 cDNA sequence. The primary combination trp2/5-LIGA and trp2/3-LIGA (Table 2) was used to amplify a 5'-deleted trp-2 cDNA sequence. Both PCR amplificates were then treated as follows: Using the mentioned restriction intersections, the fragment was cloned into the vector pLIGA160 in frame downstream from the plasmid-encoded actA signal sequence. The expression of the resulting fusion gene is controlled by the plasmid-encoded actA-promotor (Domann, et al., 1992, EMBO J. 11: 1981-1990; Gene Bank Accession: X59723). The inserted DNA sequence and its transition points to the vector were sequenced as a check. The resulting expression vector is designated pLIGA-trp2f (coded total trp-2 cDNA) or pLIGA-trp2 (coded 5'-deleted trp-2 cDNA). The latter was deposited with the DSMZ Braunschweig on 5 October 1999 as No. DSM 13072.

Example 4: Production of a Listeria Expression Vector Expressing the Human MelanA/MART-1 Gene

At the 5' and 3' end of the cDNA (Gene Bank Access Number: U06452) encoding for human tumor-associated MelanA antigen, PCR (see Table 1) was used with the primers indicated in Table 1 (melanA-5/2-LIGA, melanA/3-LIGA) to introduce a NdeI and a Bgl II recognition sequence. Using the mentioned restriction intersections, the fragment was cloned into the vector pLIGA160 in frame downstream from the plasmid-encoded actA signal sequence. The expression of the resulting fusion gene is controlled by the plasmid-encoded actA-promotor (Domann, et al., 1992, EMBO J. 11: 1981-1990; Gene Bank Accession: X59723). The inserted DNA sequence and its transition points to the vector were sequenced as a check.

Example 5: Antigen Expression in Listeria Monocytogenes

The Listeria expression vectors described in the preceding Examples 1 to 4 were introduced after amplification in the E. coli strain XL2-Blue into the L. monocytogenes strain EGD and into the attenuated mutants Δhly2 and ΔactA (Guzmann, et al., 1995, Infect. Immun. 63: 3665-3573) by electroporation. The technique used for this is sufficiently known to the practitioner. Listeria carrying the plasmid were identified by means of the plasmid-mediated erythromycin resistance. The expression of the tumor-associated antigens is then determined by use of immunological detection methods (such as immunological staining, western blot) by specific antibodies (MelanA antibodies, obtainable from the Novocastra company; tyrosinase antibodies, available from the BioTrend company, Cologne; Trp-1 antibodies, described in Thomsen, et al., 1985, J. Invest. Dermatol. 85: 169-174). It was possible to detect each of the tumor-associated antigens, i.e., the selected expression pathway was successful.

Example 7: Immunization of Mice of the Transgenic Mouse Strain HLA-A2

The Listeria expression vector pLIGA-MelanA described in the above Example 4, after amplification in the E. coli strain XL2-Blue was introduced into the L. monocytogenes strain EGD (Guzman, et al., 1995, Infect. Immun. 63: 3665-3573) by means of electroporation. These bacteria were grown over night at 37°C in BHI (brain-heart infusion) medium (manufacturer: Difco). A 1:50 dilution culture was started and the growth of the bacteria continued up to the middle log-phase. The bacteria were harvested by centrifugation at 3000xg. The cells were washed three times with PBS and resuspended in PBS. As a control, the EGD bacteria carrying the unaltered plasmid pLIGA160 were treated and cultivated the same way.

Mice of the transgenic strain HLA-A2k^b (Vitiello, et al., 1991, J. Exp. Med. 173: 1007-1015) were immunized with the bacteria resuspended in PBS (EGD-pLIGA-MelanA and EGD-pLIGA160) in the 7-day interval. The mice each received an oral application of $1x10^6$ bacteria on day 0 and $1x10^7$ bacteria on day 7, 14, 21, and so on.

The primary goal of the immunization experiment is to produce a cellular cytotoxic T-cell response. The antigen-specific activity of cytotoxic T-cells is the foundation for an efficient antitumor immune response.

The spleens of three immunized mice were removed 7 days after the last immunization, pooled together, and mechanically processed to produce a single-cell suspension. The spleen cells are restimulated with a cell line (C1R-A2k^b) transgenic to HLA-A2k^b. This cell line is stably transfected with the MelanA-antigen encoding gene and can therefore be used to stimulate the cytotoxic activity of antigen-specific T-cells. After 5-7 days, the living cells are harvested and tested for their ability to lyse the mentioned antigen-expressing target cells in the sufficiently familiar ^{51}Cr liberation experiment.

[table]
L. monocytogenes EGD | % specific lysis of the MelanA-expressing C1R-A2k^b target cells, effector:target ratio
[data, see original]
This shows the MHC Class I Melan A-restricted lysis of MelanA-expressing C1R-A2k^b target cells by lymphocytes which were primary stimulated in vivo with the recombinant L. monocytogenes EGD pLIGA-MelanA vaccine strain. 7 days after the last immunization with EGD-pLIGA-MelanA or EGD-pLIGA160 (negative control), the spleen cells were restimulated in vitro on day 5 after removal. For the in vitro restimulation, we used the mouse strain syngenic C1R-A2kb cell line, transgenic to the immunized HLA-A2k^b, which was stably transfected with the human MelanA-encoding gene. Toward the end of the culturing, the lymphocytes were tested in the ^{51}Cr liberation experiment with regard to their ability to lyse the C1R-A2k^b MelanA transfected cell line.

EGD-pLIGA160: negative control

Table 1
Primer for Amplification of the Complete Antigen-Encoding cDNA

[see original]
Table 1: Primer for Amplification of the Antigen Encoding cDNA Sequences.
Each time the recognition sequence of specific restriction enzymes that were used for the cloning of the PCR amplificate into the vector pLIGA160 is shown in bold print. The portion of a primer that binds to the complementary sequence of the mentioned c-DNA is underlined.

PCR conditions for the amplification of the mentioned c-DNA fragments:

• Initial denaturing:	3 min. at 94°C
• Cycle (40 X):	0.5 min. at 94°C
	1 min. at 55°C
	1.5 min. at 68°C
• Final polymerization:	7 min. at 68°C

Table 2
Primer for Amplification of the 5'-Shortened Antigen-Encoding cDNA
[see original]
Table 1: Primer for Amplification of the Antigen Encoding 5'-Deleted cDNA Sequences.
Each time the recognition sequence of specific restriction enzymes that were used for the cloning of the PCR amplificate into the vector pLIGA160 is shown in bold print. The portion of a primer that binds to the complementary sequence of the mentioned c-DNA is underlined.

PCR conditions for the amplification of the mentioned c-DNA fragments:

• Initial denaturing:	3 min. at 94°C
• Cycle (40 X):	0.5 min. at 94°C
	1 min. at 55°C
	1.5 min. at 68°C
• Final polymerization:	7 min. at 68°C

Example 8: Infection of Human Dendritic Cells with L. Monocytogenes EGD and the Attenuated Mutant L. Monocytogenes Δhly2

Since recombinant Listerias are to be used as a vector system in the context of an anti-melanoma vaccine in humans, it is necessary to analyze the interaction of these bacteria with the human cells of the immune system, besides studies on animal models. For this reason, the interaction of Listeria monocytogenes EGD (wild type strain) as well as additional attenuated strains with human dendritic cells (DC) was investigated. Only DC are able to initiate a primary immune response to the particular antigen after taking up an antigen vaccine (Banchereau, et al., (1998), Nature 392, pp. 245-252). For an efficient delivery of the anti-melanoma vaccine to the DC, it is therefore necessary for these cells to be infected by the Listeria. The following experiment was carried out to determine the infection efficiency: using a standard protocol (Thurner, et al., (1999), J. Immunol. Methods 223, p. 1-15), immature DC were generated in vitro from peripheral blood cells, and these were infected with the bacteria in a ratio of 1 (DC) : 5 (bacteria) (i.e., 1×10^5 DC were incubated with 5×10^5 bacteria). In order to boost the infection efficiency, centrifuging was done for 5 minutes at 1200 rpm. After one hour of incubation, 10 μg/ml of Gentamycin was added to the cultures to kill off the extra cellular bacteria. After another 3 hours, the DC were washed twice with PBS and lysed by incubation with 1% NP-40 PBS, in order to liberate the bacteria taken up

phagocytotically. To determine the bacterial count, aliquot parts of the lysate were plated on bacterial growth agar ("brain heart infusion agar"). Since each intact bacterium forms a colony on the agar after incubation, the number of intracellular bacteria is defined as the number of colony-forming units (CFU). The following Table 3 shows the outcome of the infection experiment, which proves that immature DC can be efficiently infected by Listerias.

Table 3

	L. monocytogenes EGD (wild type)	L monocytogenes Δhly2 (deletion of the Listeriolysine gene)
CFU	2.94×10^5	4.82×10^4

Example 9: Determination of the Influence of the Infection on the Phenotype of Dendritic Cells

The interaction of immature DC with bacteria can result in their maturation and thus have a positive influence on their ability to stimulate T-cells. The maturing of the DC involves changes in the expression pattern of specific surface markers, i.e., the expression of specific surface molecules which are essential to the stimulation of a cellular immune response is strengthened. This involves costimulatory molecules such as CD40, CD80, CD86 or adhesion molecules like CD54. The CD83 surface molecule is a specific marker for mature dendritic cells, although its function is not yet clear. Detection of the expression of this surface marker is done by immunofluorescence in the flow cytometer (FACS).

In order to determine the influence of Listeria infection on the phenotype of human DC, we proceeded as follows: By a standard protocol (Thurner, et al. (1999), J. Immunol. Methods 223, p. 1-15), immature DC were generated in vitro from peripheral blood cells and they were infected with L. monocytogenes EGD wild type strain bacteria in a ratio of 1 (DC) : 5 (bacteria) (i.e., 1×10^5 DC were incubated with 5×10^5 bacteria). In order to boost the infection efficiency, centrifuging was done for 5 minutes at 1200 rpm. After one hour of incubation, 10 µg/ml of Gentamycin was added to the cultures to kill off the extra cellular bacteria. The batches were then incubated for another 20 hours. As the control, parallel cultures were used, which were left uninfected. After this, the DC were harvested and washed. The cells were indirectly or indirectly [sic!] incubated with the following monoclonal antibodies: FITC-conjugated anti-HLA-DR (Becton Dickinson, Heidelberg), anti-CD54 and PE-conjugated anti-CD83 (Coulter-Immunotech, Hamburg), PE-conjugated anti-CD80 (Pharmingen, Hamburg), FITC-conjugated anti-CD40 and FITC-conjugated anti-CD86 (Cymbus Biotechnology, Dianova, Hamburg). FITC-conjugated anti-mouse-IgG (Dianova, Hamburg) was used as a secondary reagent for the anti-CD54 detection. Mouse IgG was used as the isotype control. Analysis of the expression of the surface marker on the dendritic cells was done by cytofluorometry (FACScan, Becton Dickinson company). The result is shown in Figure 5. This figure shows that the infection produced an intensified expression of specific costimulatory molecules. Furthermore, the infection brings about a maturation of the DC, visible by means of the CD83 expression. These data show that the infection of the CD with bacterial vaccine vectors has a positive effect on the phenotype of the antigen-presenting cells.

Patent Claims

1. Listeria expression vector for immunotherapy, wherein the Listeria expression vector comprises the following DNA sequences, functionally interconnected:
   (a) a promotor active in Listeria; and
   (b) a DNA sequence coding for human tyrosinase, Trp-1, MelanA/MART-1 or Trp-2.

2. Listeria expression vector per Claim 1, wherein the promotor active in Listeria is the promotor of the hly, actA, plcA, plcB or mpl gene.

3. Listeria expression vector per Claim 1 or 2, additionally containing a DNA encoding a protein, so that a fusion protein is encoded that comprises a Listeria protein and human tyrosinase, Trp-1, MelanA/MART-1 or Trp-2.

4. Expression vector per Claim 3, wherein the Listeria protein is an enzyme participating in the lysis of the host vacuoles or the migration of the host cell.

5. Expression vector per Claim 4, wherein the Listeria protein is Lysteriolysin O, PI-PLC or ActA.

6. Expression vector per Claim 3, wherein the Listeria protein comprises a signal segment of a secreted Listeria protein.

7. Expression vector per Claim 6, wherein the signal sequence comes from hemolysin ((Listeriolysin O), a phospholipase (PI-PLC), or the ActA protein.

8. Expression vector according to one of Claims 1 to 7, which comes from pKSV7, pAUL-A or pLIGA160.

9. Recombinant attenuated Listeria bacterium, containing the Listeria expression vector according to one of Claims 1 to 8..

10. Recombinant attenuated Listeria bacterium per Claim 9, which is Listeria monocytogenes.

11. Inoculant, containing a recombinant attenuated Listeria bacterium per Claim 9 or 10.

12. Use of the recombinant attenuated Listeria bacterium per Claim 9 or 10 for immunotherapy.

13. Use per Claim 12, wherein the immunotherapy is the treatment of malignant melanoma.